

The Effect of Feed Deprivation on Tissue Invasion by *Salmonella enteritidis*

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ABSTRACT A tissue culture procedure was utilized to compare tissue cell invasion by *Salmonella enteritidis* from molted and full feed hens. Three identical trials were performed in which 80-wk-old active laying hens were divided into 2 groups of 6 birds each. The molted hen group was subjected to a 14-d feed withdrawal, and the full-fed hen group was administered a standard layer ration. After feed treatment, crop, ileum, cecum, and ovary (small and large yellow follicles removed) were collected, rinsed in PBS, and placed into 50 mL of RPMI medium. The ends of intestine and crop tissues were tied to allow attachment of *Salmonella* only to the lumen surface. The RPMI medium containing 10^7 to 10^8 cfu of novobiocin and nalidixic acid-resistant phage type 13 *Salmonella enteritidis* was injected into the lumen of the intestine and crop tissues. Additionally, ovaries were incubated in 50 mL of RPMI medium containing 10^6 to 10^7

cfu of the *Salmonella enteritidis*. Tissues were incubated with *Salmonella* at 37°C for 2 h, after which tissues were placed in 50 mL of fresh RPMI medium containing 500 µg/mL of gentamicin and incubated for 5 h at 37°C to remove any *Salmonella* that had not penetrated tissues. Tissues were rinsed, stomached in 10 mL of PBS, serially diluted, and plated onto brilliant green agar containing novobiocin and nalidixic acid for *Salmonella* enumeration. *Salmonella* invasion of ovaries was reduced in tissues from molted hens in trials 1 and 2 as compared with full-fed controls (>1.2 log reduction) but not in trial 3. *Salmonella* invasion of ceca from molted hens was numerically increased in trials 1 and 2 and significantly increased in trial 3 as compared with controls (>0.8 log increase). No significant differences in *Salmonella* invasion were detected for crops and ileum. These data suggest that molting may affect invasion of tissues by *Salmonella enteritidis*.

Key words: *Salmonella enteritidis*, molting, invasion

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INTRODUCTION

During the past 10 to 15 yr, the number of cases of gastroenteritis due to *Salmonella enteritidis* (SE) infections has increased markedly in the United States and Europe (Holt et al., 1995). Epidemiological studies have attributed these episodes of SE infection to the consumption of food containing contaminated Grade A shell eggs (St. Louis et al., 1988). Since SE is invasive in poultry, it has the potential to contaminate eggs by transovarian transmission following colonization of the intestinal tract (Thiagarajan et al., 1994).

Feed deprivation is the most commonly used method by the United States layer industry to induce molting and stimulate multiple egg-laying cycles in aging hens (Bell, 1987). In 1987, an estimated 60% of laying flocks nationally were recycled (Bell, 1987). However, research has demonstrated that feed removal during forced molt decreases the resistance of hens to SE infection (Holt, 1993; Durant et al., 1999), resulting in increased severity

of infection (Holt and Porter, 1992a; Porter and Holt, 1993), increased intestinal shedding of SE (Holt and Porter, 1992a, 1993), and increased horizontal spread of infection to molted hens in neighboring cages (Holt and Porter, 1992b, 1993; Holt, 1995).

The resultant risk of SE-positive eggs appears to increase 3-fold in molted flocks during the first 10 wk after molt (USDA, 1989). Sixty percent of the approximately 300 million hens nationwide are force-molted (Bell, 1987), placing approximately 180 million hens at increased risk of SE colonization.

Physiologically, induced molting through feed withdrawal has numerous effects on the bird including a decline in body weight and a size reduction in the ovaries, oviduct, liver, and intestines. Effects on the immune system have also been suggested. The following study was performed to further investigate the mechanism of the increased susceptibility of molting hens to SE infection and to determine if tissues from feed-deprived hens can be more readily infiltrated by SE than tissues from full-fed hens in vitro.

MATERIALS AND METHODS

Tissue Collection and Preparation

In each trial, 80-wk-old actively laying specific pathogen-free Single Comb White Leghorn hens were divided

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into 2 feed treatment groups of 6 birds each: feed deprivation hens in which feed was withdrawn for 14 d and full-fed hens that received a commercial layer ration (NRC, 1994) ad libitum for 14 d. After feed treatment, crops, ilea, ceca, and ovaries were aseptically removed from each bird, rinsed with sterile PBS (pH 7.2), and placed into sterile RPMI 1640 Medium with L-glutamine (Sigma-Aldrich, St. Louis, MO). Prior to rinsing ovaries, the large and small yellow follicles were removed and the remainder of the ovary containing only small white follicles was rinsed with PBS and placed into cell culture medium. This was performed because of the absence of yellow follicles in feed withdrawal hens and a decreased incidence of *Salmonella* invasion in yellow follicles (Moore et al., 2003), allowing for a more similar comparison of tissues between the 2 groups. A 5-cm section of ileum and cecum was cut from each similar region of each tissue (1 section per bird), and the open ends of the tissue were sealed by tying them closed with sterile cotton string. The upper and lower esophagus was tied at the opening of the crops. Ligation allowed for inoculation of only the luminal surface of the tissues. Tissues in medium were maintained on ice prior to incubation.

Bacterial Preparation and Inoculation

A primary poultry isolate of SE (phage type 13, USDA, Animal and Plant Inspection Service, National Veterinary Services Laboratories, Ames, Iowa) selected for resistance to novobiocin (NO) and nalidixic acid (NA) and sensitivity to gentamicin sulfate (GS) was grown overnight in tryptic soy broth. Cell suspensions were washed twice and diluted in PBS to 10^9 cfu of SE/mL. Final dilutions of SE were made with RPMI medium. Using a syringe with a 25-gauge needle, 10^7 to 10^8 cfu of SE/mL was inoculated into the lumen of the crops (1.5 mL/crop), ilea (0.5 mL/ileum), and ceca (0.5 mL/cecum). Ovaries were placed in 50 mL of RPMI containing 10^6 to 10^7 cfu of SE/mL. All tissues were incubated in 50 mL of RPMI medium (6 tissues/50 mL of RPMI) with 5% CO₂ at 37°C for 2 h to allow for bacterial penetration of tissues. Crop, ileum, and cecum were inoculated with 10^7 cfu of SE in trials 1 and 2 and 10^8 cfu of SE in trial 3. Ovaries were inoculated with 10^7 cfu of SE in trials 1 and 2 and 10^6 cfu of SE in trial 3.

Detection of *Salmonella* Invasion

After incubation, the ligated ends of the crops and intestinal sections were excised at the ligature and opened longitudinally to allow for better contact of the antibiotic with the lumen. Tissues were rinsed with fresh medium and placed in sterile RPMI, which contained 500 µg/mL of GS. Tissues were then incubated for an additional 5 h at 37°C with 5% CO₂. The concentration and duration of GS used was determined with repeated antibiotic sensitivity testing in previous studies (Moore et al., 2003). This was done to kill off any SE present in the culture media that had not invaded the target tissue

because GS does not efficiently penetrate the tissues (Moore et al., 2003). After incubation with GS, tissues were removed from the culture media, rinsed in PBS, weighed, and individually stomached with 10 mL of sterile phosphate buffered saline. Serial dilutions (10^{-1} to 10^{-5}) of stomached samples were made and plated onto Brilliant Green Agar (Becton, Dickinson and Co., Sparks, MD) containing 25 µg/mL of NO (Sigma-Aldrich) and 20 µg/mL of NA (BGA-NO/NA, Sigma-Aldrich). Plates were incubated for 24 h at 37°C and the colony-forming units per gram of tissue were enumerated. Additionally, 1 mL of stomached material from each sample was added to 10 mL of Rappaport-Vassiliadis (Oxoid Ltd., Basingstoke, Hants., England) broth, incubated for 24 h at 37°C, and isolated into BGA-NO/NA plates. Plates were incubated for 24 h at 37°C and examined for the presence of SE colonies. Culture media in which cells were incubated with GS were also incubated in Rappaport-Vassiliadis broth and plated to assure sufficient kill of introduced SE. Further identification as SE was performed serologically using *Salmonella* O antiserum group D, factors 1, 9, 12 (Becton, Dickinson and Co.).

Tissue Viability

Additionally, tissue samples were cut from a subset of birds and stained with Trypan Blue (Sigma-Aldrich) stain at various stages of the procedure to assess cell viability. No difference in cell viability was identified between pre- and postincubation with SE, GS, or both (data not shown). Furthermore, no difference in staining was observed between feed-deprived birds and full-fed birds, suggesting similar viability of the tissues from both treatment groups (data not shown). An additional set of tissue samples were then made nonviable by incubation at 55°C for 5 min. Nonheat-treated and heat-treated tissue samples were then incubated with SE and GS to allow for identification of SE invasion. The SE recovery of heat-treated tissues was significantly reduced as compared with the nonheat-treated tissues, suggesting that differences in SE uptake were not correlated with cell viability (data not shown).

Statistical Analyses

Each trial consisted of 6 samples per tissue treatment group. Data were analyzed using SAS statistical analysis software (SAS Institute, 1999). Log colony-forming units of SE counts among treatment groups were determined by ANOVA using the GLM procedures. Statistical analyses were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

As a laying hen ages, its ability to lay eggs decreases (Cunningham et al., 1960; Etches, 1990) and continues to decline to a point at which it is no longer economically feasible to retain flock production. Induced molting reju-

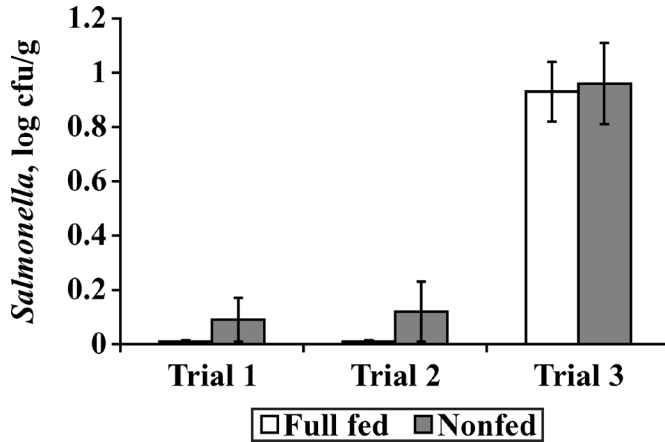


Figure 1. The penetration of crop tissues from full-fed and 14-d fasted (nonfed) hens by *Salmonella enteritidis* (SE) after a 2-h in vitro incubation. Values are means log of *Salmonella* colony-forming units per gram of crop tissue, $n = 6$ ovaries per treatment group in each trial. No significant differences were determined. Challenge dose was 10^7 cfu of SE in trials 1 and 2 and 10^8 cfu of SE in trial 3.

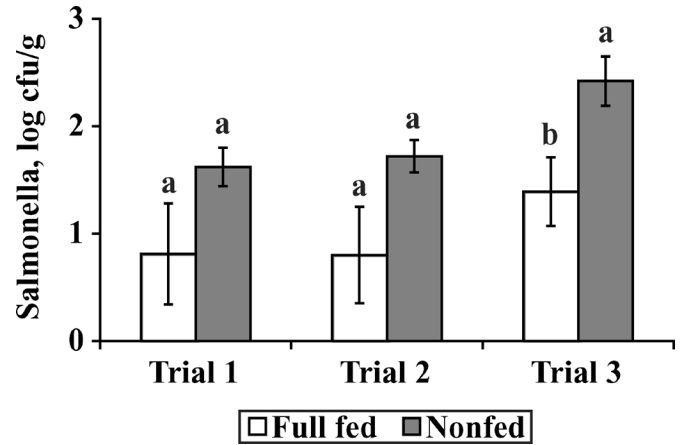


Figure 3. The penetration of cecal tissues from full-fed and 14-d fasted (nonfed) hens by *Salmonella enteritidis* (SE) after a 2-h in vitro incubation. Values are means log of *Salmonella* colony-forming units per gram of cecum, $n = 6$ ceca per treatment group in each trial. ^{a,b}Means with different letters within a trial are significantly different, $P < 0.05$. Challenge dose was 10^7 cfu of SE in trials 1 and 2 and 10^8 cfu of SE in trial 3.

venates the reproductive tract and stimulates hens at the end of a laying cycle to enter a new cycle of egg production. However, a complete and sustained regression of the oviduct along with a 25 to 30% loss of BW is required to optimize second cycle egg production. Baker et al. (1983) observed that increased BW loss correlates with increased postmolt egg production and egg quality. In the United States, typically feed is removed until the hens drop 25 to 30% of their BW. While feed removal provides the benefit of extending the effective egg-laying life of the flock, it may have negative effects on the ability of the intestines to prevent infection. Deficient diets have been shown to diminish humoral immunity (Ben-Nathan et al., 1977, 1981; Gross and Newberne, 1980) and cell-mediated immunity (Chandra, 1974, 1990;

Depasquale-Jardieu and Fraker, 1979) in mammals and birds, and to increase corticosteroid levels (Brake et al., 1979; Etches et al., 1984).

Responses of feed-deprived molted birds to SE infection were also affected; hens exposed to an exogenous source of SE concomitantly during molt induction exhibited a more severe infection as compared with their unmolted counterparts. The intestinal shed rate was higher in feed-deprived hens (Holt and Porter, 1992a, 1992b; Holt et al., 1995), and these hens also shed more organisms (Holt and Porter 1992a,b; Holt, 1993; Holt et al. 1994, 1995) and exhibited significantly more intestinal inflammation, primarily in the colon and cecum, because of the infection (Holt and Porter, 1992a; Porter and Holt,

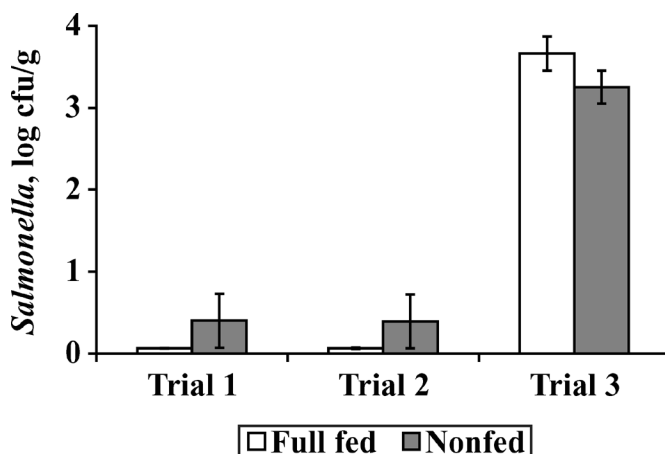


Figure 2. The penetration of ileal tissues from full-fed and 14-d fasted (nonfed) hens by *Salmonella enteritidis* (SE) after a 2-h in vitro incubation. Values are means log of *Salmonella* colony-forming units per gram of ileum, $n = 6$ ilea per treatment group in each trial. No significant differences were determined. Challenge dose was 10^7 cfu of SE in trials 1 and 2 and 10^8 cfu of SE in trial 3.

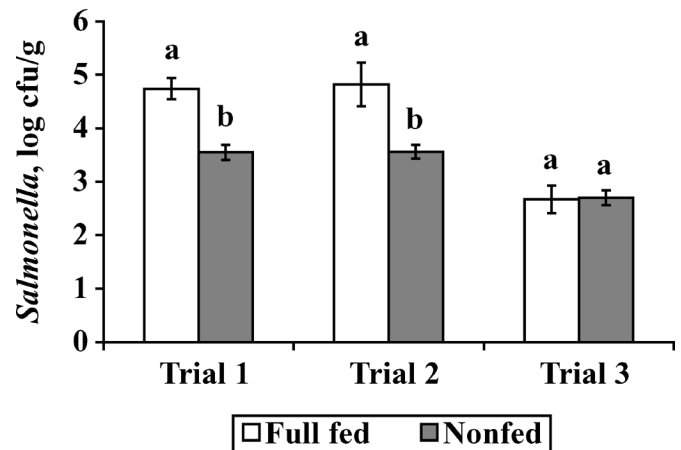


Figure 4. The penetration of ovarian tissues from full-fed and 14-d fasted (nonfed) hens by *Salmonella enteritidis* (SE) after a 2-h in vitro incubation. Values are means log of *Salmonella* colony-forming units per gram of ovarian tissue, $n = 6$ ovaries per treatment group in each trial. ^{a,b}Means with different letters within a trial are significantly different, $P < 0.05$. Challenge dose was 10^7 cfu of SE in trials 1 and 2 and 10^6 cfu of SE in trial 3.

1993; Holt et al., 1995; Arnold and Holt, 1996; Macri et al., 1997). Additionally, hens were 100- to 1,000-fold more susceptible to an SE infection (Holt, 1993; Holt et al., 1994), and readily transmitted SE horizontally to birds in adjacent cages (Holt, 1995) and to birds in cages a distance away (Holt et al., 1998). Molting also caused the recurrence of a previous SE infection (Holt and Porter, 1993) indicating that the procedure can significantly affect an SE infection at different times in the infection cycle. The SE penetration of crops was unaffected by feed withdrawal in any of the trials (Figure 1). The SE penetration of ilea was numerically increased in nonfed hens in trials 1 and 2 (Figure 2). The penetration of ceca by SE was significantly increased by feed withdrawal in trial 3 ($P < 0.05$) and numerically increased in trials 1 and 2 (Figure 3). These data may suggest that there is a difference in the ability of SE to invade tissues from nonfed hens as compared with full-fed hens in vitro.

Similar in vivo models for detection of pathogen invasion of intestinal and esophageal tissues have been developed for birds (Pal et al., 1968; Aabo et al., 2000) and mammals (Giannella et al. 1973; Fedorka-Cray et al., 1995). These models utilize sectioning of tissues with ligature similar to methods used in this study, although general anesthesia was used to place the ligations in live animals and ligated tissues were not excised till after pathogen invasion. Although the in vivo methods are arguably better models for determining the mechanism of *Salmonella* invasion, large-scale studies with multiple treatment groups would be difficult to perform. Additionally, similar in vivo techniques would not be possible with ovarian tissues. Another in vivo method for measuring ovarian invasion of *Salmonella* was performed by Thiagarajan et al. (1994), but the study exclusively examined *Salmonella* invasion of granulosa cells. The current study allows for the simultaneous examination of multiple treatments in multiple organs.

These data support in vivo findings and suggest that intestinal sections from feed-deprived hens are more susceptible to SE infiltration in vitro. However, SE invasion of ovaries was significantly reduced in nonfed hens in trials 1 and 2 ($P < 0.05$) but not in trial 3 (Figure 4), suggesting that ovaries from full-fed hens are more susceptible to *Salmonella* invasion than ovaries from molted hens. These findings are contrary to in vivo findings that show that fasted hens have a higher incidence of ovarian infection than full-fed hens. Although difficult to explain from current data, these finding may involve active recruitment of follicles and yolk deposition in active laying hens. These data do suggest that fasting and ovarian regression do not exert a direct effect on *Salmonella* invasion of ovaries and that an alternate mechanism is involved in the susceptibility of fasted hens to ovarian invasion by *Salmonella*.

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